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Herpesvirus Papio 2 Encodes a Virion Host Shutoff Function

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Infection of baboons with herpesvirus papio 2 (HVP-2) produces a disease that is similar to human infection with herpes simplex viruses (HSV). Molecular characterization of HVP-2 has demonstrated that the virion contains a factor which rapidly shuts off host cell protein synthesis after infection. Reduction of host cell protein synthesis occurs in parallel with the degradation of mRNA species. A homolog of the HSV virion host shutoff (vhs) gene was identified by Southern and DNA sequence analysis. The sequence of the HVP-2 *vhs* gene homolog had greater than 70% identity with the *vhs* genes of HSV 1 and 2. Disruption of the HVP-2 *vhs* open reading frame diminished the ability of the virus to shut off protein synthesis and degrade cellular mRNA, indicating that this gene was responsible for the vhs activity. The HVP-2 model system provides the opportunity to study the biological role of vhs in the context of a natural primate host. Further development of this system will provide a platform for proof-of-concept studies that will test the efficacy of vaccines that utilize vhs-deficient viruses.
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INTRODUCTION

Herpesvirus papio 2 (HVP-2) infection of baboons represents a surrogate primate model system to study the biology of herpes simplex virus (HSV) infection of humans. HVP-2 is associated with oral and genital lesions that resemble HSV infections of humans. Male baboons develop erythema of the penis with papules or pustules that develop into small ulcerative lesions. Female baboons have been observed with ulcerative lesions on the vulvar tissues. Juvenile animals were found to have primarily oral lesions (Levin *et al.,* 1988). Acquisition of genital infection is primarily associated with the onset of sexual activity in baboons. Furthermore, one study provided evidence that the virus could spontaneously reactivate from latency, a pattern consistent with HSV infection of humans (Martino *et al.,* 1998).

Early attempts to characterize virus isolated from infected baboons resulted in designation of the virus as SA8, a virus first isolated from African green monkeys (Levin *et al.,* 1988). Subsequent analysis of three viral glycoprotein genes (glycoproteins B, D, and J) confirmed that the herpesvirus associated with the baboon disease was closely related to other members of the alphaherpesvirus family (HSV-1, HSV-2, herpes B virus). Although closely related, the isolates represented a new virus associated with baboons, which was designated as herpesvirus papio 2 (HVP-2) (Eberle *et al.,* 1995). Subsequent serological analysis has determined that over 90% of wild-caught baboons were found to have anti-HVP-2 titers, confirming that baboons are a natural host for HVP-2 (Eberle *et al.,* 1997).

A hallmark of alphaherpesvirus infection is the shutoff of host protein synthesis during infection (Fenwick, 1984). Shutoff of host macromolecular synthesis occurs through two independent mechanisms in HSV-infected cells. Early phase shutoff is mediated by a 58-kDa phosphoprotein encoded by the viral UL41 gene designated the virion host shutoff (vhs) protein (Read and Frenkel, 1983; Kwong *et al.,* 1988, McGeoch *et al.,* 1988; Read *et al.,* 1993). During infection, the virion tegument (containing vhs) is released into the cytoplasm of the host cell. Cytoplasmic vhs is associated with the degradation of mRNA (Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987; Strom and Frenkel, 1987; Oroskar and Read, 1989). Degradation of cellular mRNA shifts the translation apparatus from host to viral mRNA and facilitates the expression of different classes of viral genes. A secondary phase shutoff is dependent on the immediate-early protein ICP27 (Nishioka and Silverstein, 1978; Fenwick and Clark, 1982; McMahan and Schaffer, 1990; Hardwicke and Sandri-Goldin, 1999).

The exact mechanism of vhs activity has not been determined. Several studies have suggested that vhs is an endonuclease. First, cytoplasmic extracts prepared from HSV-infected cells or extracts prepared from HSV virions contain a vhs-dependent RNase activity (Krikorian and Read, 1991; Sorenson *et al.,* 1991; Karr and Read, 1999; Zelus *et al.,* 1996). Second, vhs induces cleavage of mRNAs when expressed as the only viral protein in a rabbit reticulolysate system (Zelus *et al.,* 1996; Elgadi *et*

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al., 1999a,b). Antibodies to vhs protein block endo-ribonucleolytic activity *in vitro* (Zelus *et al.,* 1996). Finally, vhs has homology to the fen-1 family of endonucleases (Doherty *et al.,* 1996). *In vitro* studies have shown that vhs induces endonucleolytic cleavages which cluster toward the 5′ end of the mRNA (Zelus *et al.,* 1996; Karr and Read, 1999; Elgadi *et al.,* 1999a,b). Interestingly, a recent study by Lu and co-workers (2001a) using a mutant vhs has demonstrated that the protein may direct initial cleavage events and play a role in degradation of mRNA containing IRES elements.

Two studies have demonstrated the potential role of cellular factors in vhs function. Expression of vhs in *Saccaromyces cerevisiae* did not result in any vhs-dependent endoribonuclease activity (Lu *et al.,* 2001b). However, activity was detected when the yeast extracts were mixed with rabbit reticulocyte lysates. These studies indicated that vhs function is dependent on one or more mammalian factors. In a second study, Feng and co-workers (2001) demonstrated that vhs bound to the human translation initiation factor, eIF4H, in both two-hybrid and co-immunoprecipitation assays. The results of these studies suggest that vhs is targeted to regions of translation initiation through interaction with eIF4H.

Several studies using the mouse model of infection have demonstrated that vhs is an important virulence factor. For example, Becker and co-workers (1993) demonstrated that vhs-defective viruses lack intraperitoneal pathogenicity in newborn mice. Strelow and Leib (1995) demonstrated that vhs-defective viruses did not grow efficiently in mouse cornea and brains. Furthermore, vhs-deficient viruses are not efficient at establishment and reactivation from latency in mouse trigeminal ganglia. The attenuated virulence associated with vhs-deficient viruses makes them attractive vaccine candidates. Interestingly, mice immunized with an HSV-1 strain deficient in vhs activity are protected against corneal challenge with virulent HSV-1 in a mouse model of infection (Walker *et al.,* 1998). These studies have been further extended in the mouse model to show the enhanced protection against wild-type HSV challenge in animals vaccinated with a recombinant HSV-1 with deletions in both vhs and ICP8 (Geiss *et al.,* 2000).

To develop the HVP-2 model system, we have initiated a careful comparison of the molecular biology of this virus relative to human simplex viruses. The results of the studies presented here have demonstrated that HVP-2 encodes a virion host shutoff function that is closely related to the vhs encoded by HSV-1. These studies further demonstrate the close molecular relationship between HVP-2 and HSV. The HVP-2 model provides a surrogate system to complement and extend our understanding of the biology of vhs. Future studies can test the efficacy of vhs-deficient virus vaccines in the context of a nonhuman primate system.

RESULTS

HVP-2 infection results in a shutoff of host protein synthesis

The reduction of host cell protein synthesis is a hallmark of herpes simplex virus infection (Fenwick, 1984). To determine whether host protein synthesis is reduced during HVP-2 infection, [^{35}S]methionine-labeled extracts from infected and mock-infected Vero cells were prepared at varying times postinfection and analyzed on denaturing polyacrylamide gels. Comparison of mock- and virus-infected extracts revealed that HVP-2 infection resulted in a dramatic reduction in host cell protein synthesis as early as 2 h postinfection (Fig. 1A). Host cell protein synthesis was further reduced at 3 and 4 h postinfection. The appearance of several new protein species is likely due to the expression of viral genes as addition of actinomycin D blocks the appearance of these bands (data not shown). The inhibition of host protein synthesis early in HVP-2 infection is similar to the reduction of protein synthesis observed in HSV-1-infected cells (Fig. 1A).

To determine the species-specificity of the HVP-2 protein shutoff function, [^{35}S]methionine-labeled extracts were prepared from baboon, human, and mouse cell lines 4 h postinfection. Extracts were separated on denaturing polyacrylamide gels and visualized by autoradiography. Examination of the autoradiograms demonstrated that there was a reduction in host cell protein synthesis in primary baboon fibroblasts, HepG2, and NIH 3T3 cells (Fig. 1B). The observation that HVP-2 induced a shutoff of host cell protein synthesis in primary baboon fibroblasts demonstrates that this process occurs in cells isolated from the natural host. Therefore, shutoff is a process that is likely to occur during natural infection and is not the result of an artifact of Vero cell infection. Shutoff of host protein synthesis was also observed in human liver cells (HepG2) and mouse fibroblasts (NIH3T3). Therefore, the HVP-2 host shutoff function is active in cell types from different species. This observation indicates that the shutoff of host protein synthesis by HVP-2 is likely to proceed by a conserved mechanism that can function in cells from a variety of species.

Shutoff of protein synthesis is associated with the HVP-2 virion

The decrease in host cell protein synthesis during HVP-2 infection could be due to the action of a nascent viral protein(s), the activity of an induced or activated cellular protein, or the direct action of a virion protein. To determine whether the shutoff function is mediated by a virion-associated factor, Vero cells were treated with actinomycin D to prevent nascent viral and cellular transcription. Cells were infected at an increasing multiplicity of infection (m.o.i.) with HVP-2. After 3 h, the cells were

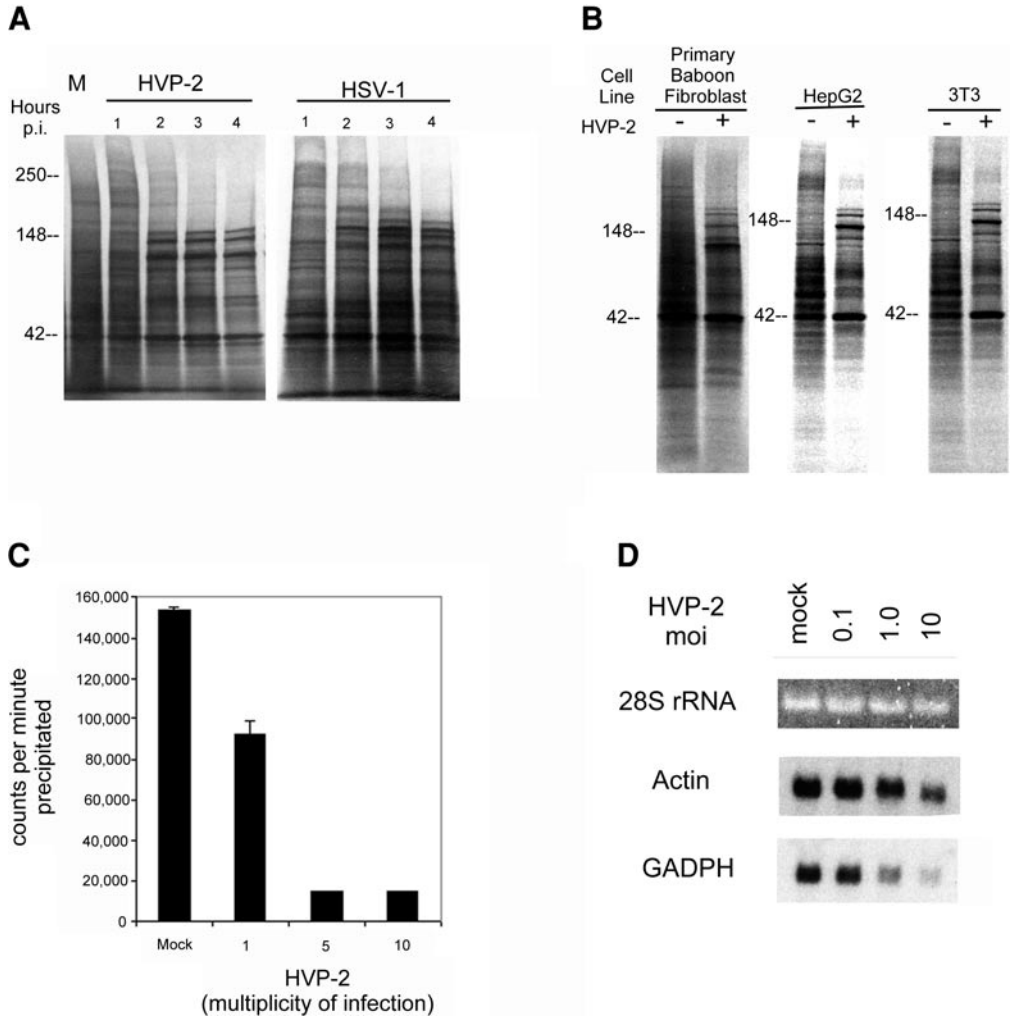

FIG. 1. HVP-2 contains a virion host shutoff activity. (A) Shutoff of host protein synthesis in Vero cells infected with HVP-2 or HSV-1. Cell monolayers were mock-infected or infected at a multiplicity of infection (m.o.i.) of 10 plaque forming units per cell with HVP-2 strain 860 or HSV-1 strain KOS. One hour prior to harvest the cells were rinsed with methionine-free minimal essential medium (MEM) and pulse labeled with [^{35}S]methionine. Cells were harvested at varying times postinfection by addition of SDS-containing lysis buffer. Samples from equivalent numbers of cell were resolved on 4–20% denaturing polyacrylamide gels and visualized by autoradiography. Note the decrease in number and intensity of protein species relative to the mock-infected lanes. Hours p.i. represent hours postinfection. The numbers along the left side of the gel correspond to molecular weight in kiloDaltons. (B) HVP-2 host shutoff activity is not restricted to Vero cells. Primary baboon fibroblasts, HepG2, and 3T3 cells infected with HVP-2. Note the decrease in number and intensity of protein species relative to the mock-infected lanes. (M) represents mock-infected lane. Hours p.i. represent hours postinfection. The numbers along the left side of the gel correspond to molecular weight in kiloDaltons. (C) HVP-2 vhs activity is associated with the virion. Vero cell monolayers were treated with actinomycin D and infected with HVP-2 or mock-infected. Three hours postinfection the samples were washed and incubated with methionine-free MEM containing [^{35}S]methionine and actinomycin D for 1 h. Cells were scraped into PBS and lysed by successive freeze-thaw cycles. The amount of labeled protein was quantitated by TCA precipitation and analyzed by liquid scintillation. Note that the addition of increasing amounts of virus decreases the amount of protein synthesis in the presence of actinomycin D. This indicates that viral gene expression is not required for shutoff of host protein synthesis and that the virion contains the vhs activity. (D) HVP-2 infection results in a decrease in levels of cellular mRNA. To determine whether HVP-2 results in a decrease of cellular messages, total RNA was isolated from infected- and mock-infected Vero cells 4 h postinfection. Equivalent amounts of samples were analyzed by Northern analysis using β-actin and GAPDH probes. The relative levels of 28S rRNA were confirmed by staining gels with ethidium bromide. The Northern blot demonstrates that increasing the m.o.i. enhances the degradation of both mRNA species. HVP-2 m.o.i. represents the HVP-2 multiplicity of infection.

labeled in media containing [^{35}S]methionine for 1 h, harvested, and lysed. The amount of labeled proteins was quantitated by trichloroacetic acid (TCA) precipitation. The results of these assays are shown in Fig. 1C. Clearly, HVP-2 infection effectively inhibited cell protein synthesis in the presence of the transcriptional inhibitor actinomycin D. Furthermore, the effect observed was dosage dependent with greater levels of shutoff at 5–10 m.o.i. relative to 1.0 m.o.i. Infection of cells with at a level of 1.0 m.o.i. resulted in levels of protein synthesis that were approximately 60% of uninfected levels. Infection with a higher m.o.i. of virus was associated with an approximately 90% inhibition of host cell protein synthesis. To confirm that the actinomycin D treatment was

effective, equivalent amounts of extracts from mock and treated cells were resolved on 4–20% SDS–PAGE gels and visualized by autoradiography. The results demonstrated that the protein species remained comigrated with cellular proteins in mock-treated cells and that there was an absence of proteins that typically appear early during viral infection (data not shown). Therefore, active levels of viral gene expression are effectively blocked by actinomycin D. Inhibition of protein synthesis in the absence of active transcription indicates that synthesis of viral gene products is not required for shutoff of host protein synthesis. Rather, the shutoff activity is brought in by the infecting virus and is therefore virion-associated. Furthermore, the magnitude of the decrease in protein synthesis was related to the amount of input virus, i.e., the phenomenon was m.o.i. dependent. These results indicate that inhibition of host protein synthesis is the result of a factor that is delivered by the HVP-2 virion and is not the result of the expression of viral genes. Taken together, these results clearly demonstrate that HVP-2 contains a virion host shutoff function.

HVP-2 infection induces degradation of host mRNA

Infection of cells with HSV-1 has been shown to induce the degradation of mRNA in a vhs-dependent manner (Kwong and Frenkel, 1987; Oroskar and Read, 1989). To determine whether HVP-2 infection induces degradation of host cell mRNA, total RNA was isolated from infected and mock-infected Vero cells 4 h postinfection. The levels of specific mRNAs were determined by Northern blot analysis using probes for mouse glyceraldehyde phosphate dehydrogenase (GAPDH) and mouse β-actin. The results of these studies are shown in Fig. 1D. Equivalent amounts of RNA were loaded as demonstrated by the levels of 28S RNA in ethidium bromide stained gels. In the case of both GAPDH and β-actin probes, the level of message present at 0.1 m.o.i. was similar to the level of message present in mock-infected cells. However, as the multiplicity of infection was increased to 1.0 and 10, there was a corresponding decrease in the level of message for both GAPDH and β-actin. The levels of the respective RNA species were quantitated by phosphorImager analysis. A 10 m.o.i. infection with HVP-2 resulted in a reduction of GAPDH message to levels of only 20% of the mock-infected samples, while β-actin was reduced to levels of approximately 10% of the mock-infected samples. Therefore, infection of cells with HVP-2 results in a dose-dependent degradation of host cell mRNA species, a phenomenon associated with vhs function in HSV-infected cells.

The HVP-2 genome contains a UL41 homolog

The results of protein synthesis and Northern blot analysis indicate that HVP-2 may contain a homolog of the HSV-1 UL41 gene that encodes the vhs protein. The physical map of the HVP-2 genome is colinear with HSV (J. E. Bigger and D. W. Martin, unpublished data). DNA sequence analysis of the ends of a 4-kilobase (kb) *Bam*HI fragment of HVP-2 strain 860 cloned into pUC19 (pBam20) showed homology to genes that flank UL41, the HSV genes UL40 and UL42. The entire clone was sequenced on both DNA strands. BLAST analysis (GenBank) of the resulting DNA sequence revealed that HVP-2 contains a HSV-1 UL41 (*vhs* gene) homolog. The sequence of this gene has been deposited in GenBank (Accession No. AF294581). The open reading frame contains 481 amino acids and would encode a protein with a calculated molecular weight of 54,216 Da. Figure 2 shows the amino acid sequence of the HVP-2 vhs homolog relative to proteins from HSV-1 strain KOS and HSV-2 strain 333. HVP-2 vhs shows greater than 70% similarity with the vhs proteins of HSV-1 and HSV-2. Previous analyses of the sequences of vhs homologs from several different alphaherpesviruses have revealed the existence of four conserved domains across the vhs protein (Berthomme *et al.,* 1993). These domains are well conserved within the HVP-2 vhs homolog. Phylogenetic analysis of the HVP-2 vhs relative to vhs homologs of other alphaherpesviruses demonstrates that the HVP-2 homolog is most closely related to the vhs proteins of HSV (Fig. 3). The close relationship between the vhs genes of HVP-2 and HSV is consistent with previous studies that demonstrated that HVP-2 is closely related to HSV (Eberle *et al.,* 1995).

Disruption of the HVP-2 UL41 homolog

A recombinant virus with a disruption in the HVP-2 vhs gene was constructed to determine whether the vhs homolog is responsible for the modulation of protein synthesis in infected cells. The pBam20 clone contains portions of the UL40 and UL42 open reading frames (ORF) as well as an intact UL41 ORF (Fig. 4). A reporter gene cassette was constructed that placed the enhanced green fluorescent protein (EGFP) gene under control of the CMV immediate early promoter. This cassette was cloned into the unique Bsu36I site of pBam20 to generate pBam20-EGFP. The resulting construct had an insertion of the reporter gene cassette 60 nucleotides from the amino-terminal end of the UL41 open reading frame, thereby interrupting the vhs protein between the alanine and leucine residues at positions 20 and 21. The pBam20-EGFP insert was transferred into the HVP-2 vhs gene by marker transfer. EGFP-expressing plaques were purified three times and the recombinant, HVP-2Δvhs, was grown to high titers and analyzed to confirm the vhs deletion. HVP-2Δvhs was subsequently rescued by marker transfer with pBam20, a plasmid that contains a region of the HVP-2 genome that spans the vhs gene. The rescue virus is designated HVP-2ΔvhsR.

The structure of the recombinant virus was confirmed

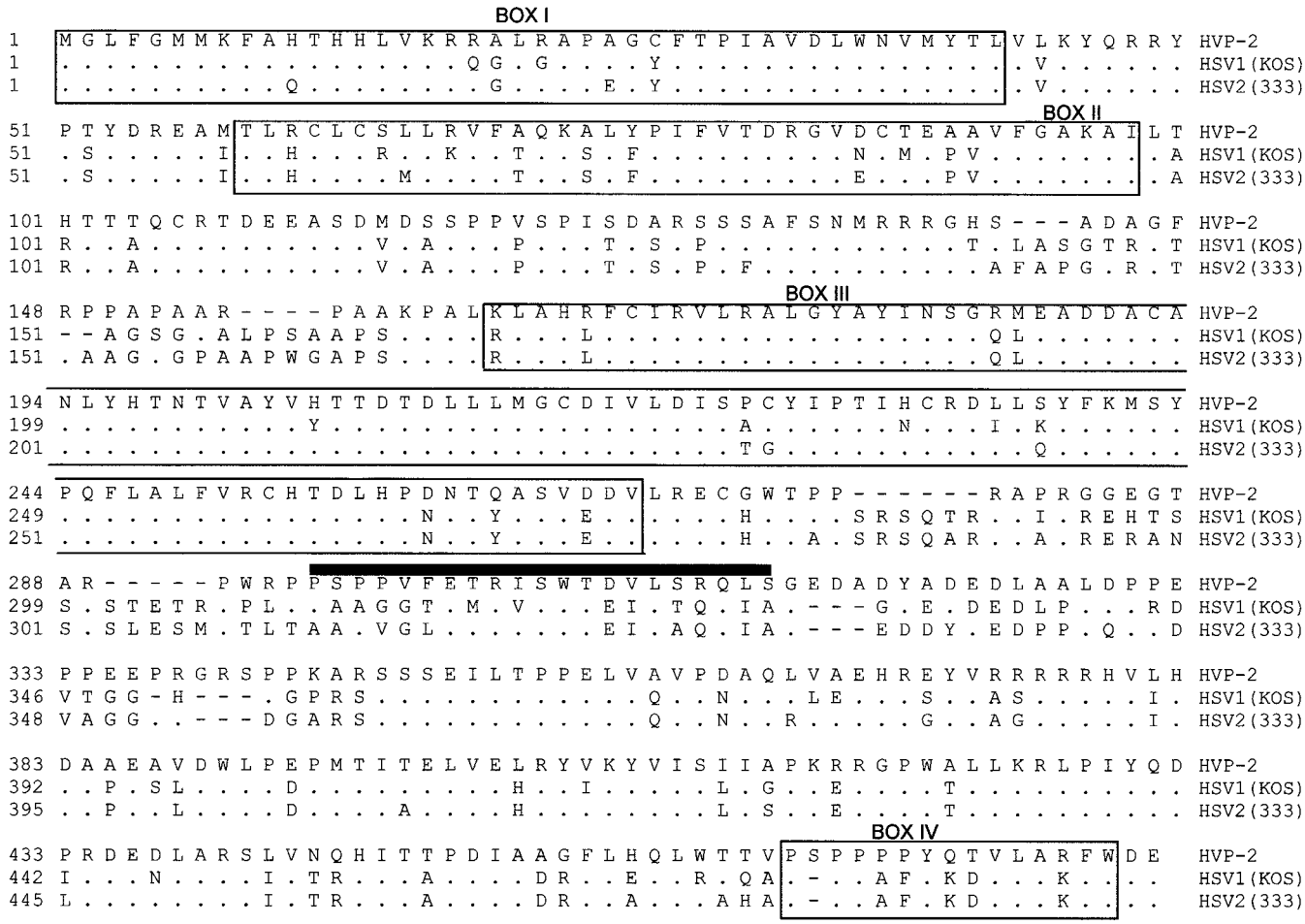

FIG. 2. HVP-2 contains a homolog of the HSV UL41 (*vhs*) gene. Sequence analysis of a 4-kb *Bam*HI fragment cloned from the HVP-2 genome revealed the presence of a homolog of the HSV *vhs* gene. The predicted reading frame of the HVP-2 vhs protein shares a high degree of conservation with vhs homologs from other alphaherpesviruses. The predicted amino acid sequence of the HVP-2 vhs protein is directly compared with the vhs proteins of HSV-1 and HSV-2. Dots indicate conserved amino acids. The positions of the Box 1, 2, 3, and 4 regions are indicated. The region important for VP16 binding in HSV-1 is designated by a bar. Note the high degree of amino acid conservation between the vhs homologs of the respective viruses.

by Southern blot analysis of viral DNA isolated from wild-type HVP-2, HVP-2Δvhs, and HVP-2ΔvhsR. A schematic of the vhs locus is shown in Fig. 4A. The presence of the EGFP insert in HVP-2Δvhs was confirmed by Southern analysis of *Bam*HI-cut DNA hybridized with the EGFP gene as a probe (Fig. 4B). As expected, neither wild-type HVP-2 or HVP-2ΔvhsR demonstrated any hybridization to the EGFP probe. The vhs locus was visible as a 4.1-kb *Bam*HI fragment in wild-type HVP-2 and HVP-2ΔvhsR DNA by Southern analysis using pBam20 as a probe (Fig. 4B). In contrast, digestion of HVP-2Δvhs with *Bam*HI demonstrated that the vhs locus is disrupted due to a *Bam*HI site donated by the reporter gene cassette, resulting in the generation of 4- and 2.2-kb *Bam*HI

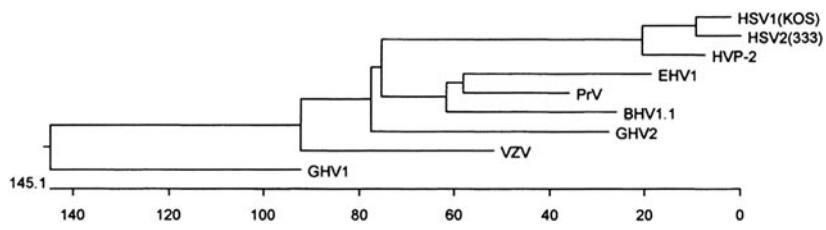

FIG. 3. Phylogenetic analysis of the HVP-2 vhs protein. Phylogenetic analysis of alphaherpesvirus homologs was performed using Clustal analysis (DNAStar) to determine the relationship between alphaherpesvirus vhs homologs. Note that the HVP-2 vhs homolog is most closely related to vhs homologs of HSV-1 and HSV-2.

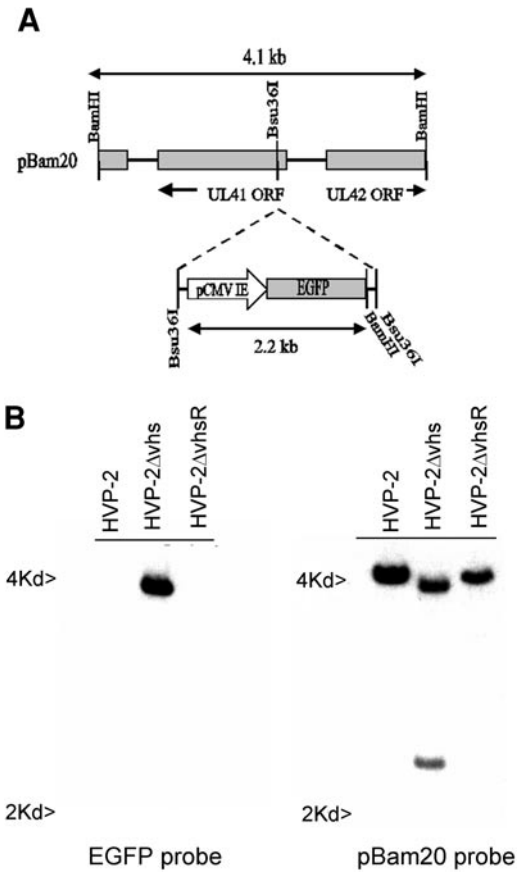

FIG. 4. Construction of HVP-2Δvhs and HVP-2ΔvhsR. (A) Physical map of the HVP-2 Bam20 fragment. The relative position of the CMV-EGFP cassette insertion in the vhs gene locus as well as the size of the respective restriction fragments are shown. (B) Southern analyses of wild-type HVP-2, HVP-2Δvhs (vhs knock-out virus), and HVP-2ΔvhsR (vhs rescue) were performed to confirm the identity of the recombinant virus. Orientation of the inserted fragment was confirmed by restriction and Southern blot analyses. Note that hybridization with a CMV-EGFP probe produced a positive signal only in HVP-2Δvhs. Hybridization with a Bam20 probe further demonstrates the disruption in the *vhs* locus relative to wild-type HVP-2 and HVP-2ΔvhsR.

bands (Fig. 4B). Taken together, the results of the Southern analysis indicate the vhs gene has been successfully interrupted in HVP-2Δvhs and successfully restored in HVP-2ΔvhsR.

HVP-2 vhs activity is not required for *in vitro* replication

The results of several experiments demonstrated that the product of the HVP-2 vhs gene is not required for *in vitro* replication. First, the ability to construct HVP-2Δvhs in a noncomplementing cell line indicates that this viral gene is dispensable for growth *in vitro*. Both wild-type and mutant viruses generate a vigorous plaque after 3 days. However, the morphology of the plaques generated by HVP-2Δvhs differed from the plaques produced by wild-type HVP-2 and HVP-2ΔvhsR on Vero cells (not shown). Plaques generated by wild-type HVP-2 and HVP2ΔvhsR on Vero cells do not typically show a clear area in the center. In contrast, plaques generated by HVP-2Δvhs demonstrate greater cell rounding in the center of the plaque which leads to a larger area in the center of the plaque where cells have detached from the surface. Second, a plaque yield assay was performed to compare the relative replication rates between wild-type HVP-2 and HVP-2Δvhs. This assay measures the burst size or number of plaque forming units released per plaque. The results in both Vero cells and primary baboon fibroblasts demonstrated that disruption of the vhs locus results in a nearly 2 log reduction in viral replication (data not shown).

Finally, a single-step growth curve was performed to quantitate the growth of the HVP-2Δvhs relative to wild-type HVP-2. Previous studies have determined that the HVP-2 growth cycle occurs in approximately 18 h (J. E. Bigger and D. W. Martin, unpublished data). Vero cells were infected at a m.o.i. of 2 in duplicate with wild-type HVP-2, HVP-2Δvhs, or HVP-2ΔvhsR and harvested at varying times postinfection. Infected cells were disrupted by successive freeze-thaw cycles and the levels of infectious virus in the supernatants were determined by plaque analysis. The results of this analysis demonstrated that HVP-2Δvhs replicated with slightly delayed kinetics relative to wild-type virus (Fig. 5). Comparison of the replication curves revealed that maximal levels of HVP-2Δvhs were approximately 1 log lower than wild-type virus at 20 h postinfection. Interestingly, the replication of HVP-2ΔvhsR displayed an intermediate pattern of replication. Although this virus replicated better than HVP-2Δvhs at 20 h postinfection, the rescue virus did not replicate as well as the wild-type isolate. We hypothesize that the difference in replication may be due to the effect

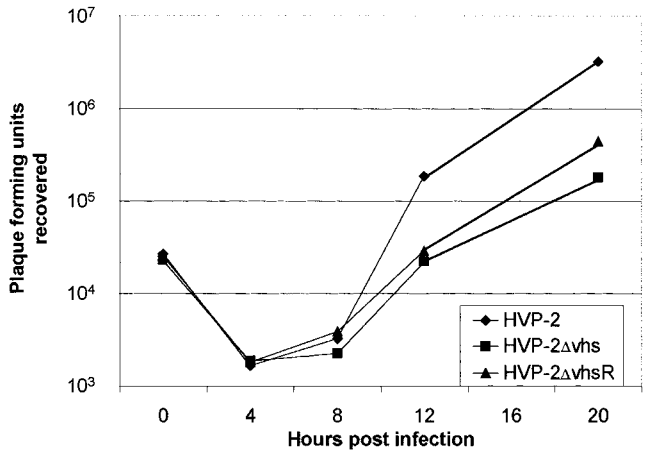

FIG. 5. HVP-2 vhs is dispensable for *in vitro* replication. Analysis of single-step growth kinetics in Vero cells. Vero cells were infected at 2 PFU/cell and harvested by freeze-thaw at varying times postinfection. Supernatants were titered on Vero cells. Although the HVP-2 vhs gene is dispensable for replication *in vitro*, HVP-2Δvhs does not replicate as efficiently as wild-type HVP-2. The replication of the rescue virus, HVP-2ΔvhsR, displays an intermediate phenotype (see text).

of multiple rounds of replication acquired during generation of the knock-out and rescue virus in Vero cells relative to the original, low-passage wild-type isolate. Future studies that involve the disruption and rescue of other HVP-2 gene products will isolate wild-type plaques in parallel with recombinant viruses to compare the replication of viruses subjected to equivalent rounds of *in vitro* replication. Taken together, these studies demonstrate that although the vhs gene is dispensable for HVP-2 replication *in vitro*, optimal replication requires the presence of functional HVP-2 vhs. Future studies will test the role of the HVP-2 vhs gene during *in vivo* infection. *In vivo* studies will require the use of a vhs knock-out virus that contains a small mutagenesis cassette to avoid any variables imposed by a large mutagenesis cassette that encodes a reporter gene.

The HVP-2 UL41 homolog encodes vhs activity

To determine whether disruption of the HVP-2 *vhs* locus affects the shutoff of host cell protein synthesis, infected cells were transiently labeled with [^{35}S]methionine and the extracts were analyzed by SDS–PAGE. The results of this assay are shown in Fig. 6A. Infection with wild-type HVP-2 results in a significant decrease in host cell protein synthesis at 4 h postinfection when compared to the mock-infected samples. Infection with HVP-2Δvhs did not result in a significant level of shutoff of host cell protein synthesis. The slight reduction observed is most likely the result of the simple competition of viral and cellular messages for the translation apparatus. However, the overall distribution of protein synthesis is very similar to that observed in mock-infected cells. Interestingly, the pattern of protein synthesis in HVP-2ΔvhsR was identical to the pattern observed in cells infected with wild-type HVP-2. Therefore, the HVP-2 vhs gene is required for efficient shutoff of host cell protein synthesis. We have shown earlier that infection of cells with wild-type HVP-2 induces the degradation of host cell mRNA (Fig. 1D). Therefore, Northern analysis was performed to determine whether the HVP-2 vhs gene product was responsible for the decrease in levels of cellular mRNA. Total RNA was isolated from cells infected with wild-type HVP-2, HVP-2Δvhs, HVP-2ΔvhsR, or mock-infected cells and analyzed to determine the relative levels of GAPDH and β-actin messages by Northern analysis (Fig. 6B). Infection with both wild-type HVP-2 and HVP-2ΔvhsR resulted in a significant reduction in the levels of both β-actin and GAPDH mRNA. In contrast, infection with HVP-2Δvhs did not cause a significant reduction in the levels of mRNA and the general levels were similar to the signals observed in mock-infected cells (Fig. 6B). Quantitation by phosphorImager analysis revealed that infection with wild-type HVP-2 and HVP-2ΔvhsR reduced the β-actin mRNA levels to 23 and 10% of mock-infected levels, respectively. In addition, the GAPDH mRNA levels

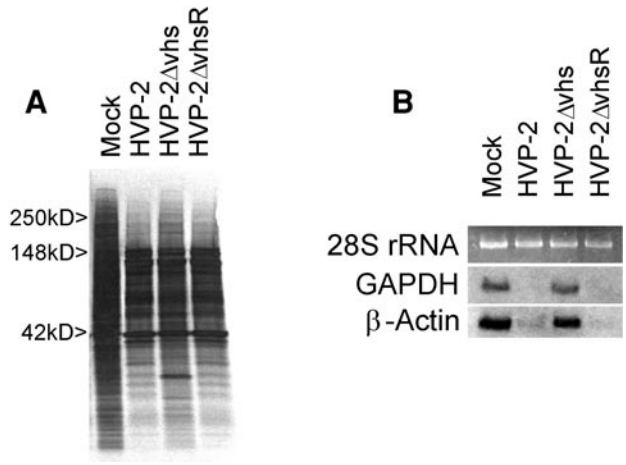

FIG. 6. The HVP-2 vhs gene is essential for shutoff activity. Deletion of the vhs gene restores host protein synthesis in infected cells. (A) Analysis of protein synthesis. Vero cells were infected with HVP-2, HVP-2Δvhs, HVP-2ΔvhsR, or mock-infected for 4 h. One hour prior to harvest the cells were radiolabeled with MEM containing [^{35}S]methionine. Cells were harvested in SDS–Tris glycine sample buffer. Equivalent amounts of extract were run on 4–20% SDS–PAGE and visualized by autoradiography. The gel is representative of two independent experiments. Numbers along the vertical axis represent the molecular weight in kiloDaltons. Note that disruption of vhs results in levels of protein synthesis similar to mock-infected cells and that restoration of the wild-type vhs gene in HVP-2ΔvhsR generates a pattern of protein synthesis identical to wild-type HVP-2. (B) Deletion of the vhs gene restores stability to host mRNA species. Northern analysis of total RNA extracted from Vero cells infected with wild-type virus, HVP-2Δvhs, HVP-2ΔvhsR, or mock-infected 4 h postinfection. Levels of total RNA were determined by ethidium bromide staining to visualize 28S RNA. mRNA was detected using mouse probes against β-actin and GAPDH. Note that disruption of the vhs gene reading frame results near wild-type levels of mRNA and that restoration of the wild-type vhs locus in HVP-2ΔvhsR results in a reduction of host mRNA as seen in cells infected with wild-type HVP-2.

were reduced to 12 and 19% the levels of observed in mock-infected cells. In contrast, cells infected with HVP-2Δvhs maintained high levels of β-actin mRNA (95%) and GAPDH mRNA (81%) relative to the levels found in mock-infected cells. Therefore, these results clearly demonstrate that the vhs gene is clearly required for both the shutoff of host protein synthesis and the reduction in host mRNA levels.

DISCUSSION

The results of these studies have demonstrated that HVP-2 encodes a virion host shutoff function. First, infection of cells with HVP-2 resulted in a decrease in host cell protein synthesis. In addition, the shutoff activity was associated with the HVP-2 virion. Second, the decrease in host protein synthesis was accompanied by degradation of cellular mRNA. Third, analysis of the HVP-2 genome revealed a homolog of the HSV UL41 (*vhs*) gene. The HVP-2 homolog maintains greater than 70% homology with the UL41 genes from HSV. Finally, a recombi-

nant virus with a disruption in the HVP-2 *vhs* gene lost the ability to shut off host protein synthesis and degrade cellular mRNA. Subsequent marker transfer of a wild-type locus back into the virus restored the ability of the virus to mediate shutoff of host protein synthesis. The vhs gene was dispensable for *in vitro* replication, although levels of virus were reduced relative to wild-type HVP-2. Taken together, these results demonstrate that HVP-2 encodes a virion host shutoff function that is closely related to the vhs protein encoded by HSV.

The identification of an HVP-2 vhs homolog is significant for several reasons. First, characterization of the *vhs* gene of HVP-2 provides further evidence of the close molecular relationship between HVP-2 and the human simplex viruses. Second, the identification of a closely related *vhs* gene in HVP-2 provides a surrogate primate system to study the *in vitro* and *in vivo* biology of vhs. Finally, the identification of a vhs homolog in HVP-2 will provide a proof-of-concept platform allowing for the further development of vhs-deficient viruses for use in vaccine therapy. The use of a relevant surrogate primate model to test the efficacy of these novel vaccines strategies should refine the development of these strategies and extend the results from small animal models of HSV infection.

The predicted reading frame of the HVP-2 vhs homolog maintains greater than 70% identity with HSV vhs. Berthomme *et al.* (1993) identified four domains of vhs that were highly conserved among alphaherpesviruses. These four domains are well conserved in HVP-2 vhs (Fig. 2). Most cases of divergence within these domains represent conservative changes. Everly and Read (1999), using point mutations of HSV-1 vhs/HSV-2 vhs chimeras, identified a number of residues of HSV-2 vhs (namely, R19, R22, E25, A396, and S423), which when placed individually into the HSV 1 vhs background would enhance vhs activity. HVP-2 vhs maintains conservation with HSV-2 vhs at each of these residues with the exception of positions 25 and 423. HVP-2 vhs substitutes alanine at position 25 (which is conserved with HSV-1 vhs) and an alanine at position 423. Finally, the sequence conservation between HVP-2 and HSV vhs proteins suggests that the protein–protein interactions observed with HSV-1 vhs may be conserved in the HVP-2 system. For example, HSV-1 vhs has been shown to interact with VP16 (Smibert *et al.,* 1994). Presumably, this interaction modulates vhs activity during late times of infection. We have previously identified a VP16 homolog in HVP-2 (GenBank Accession No. AF294740). Although this product has not yet been characterized, it will be interesting to determine whether the HVP-2 vhs interacts with HVP-2 VP16. The domain where HSV-1 vhs interacts with VP16 has been mapped to amino acids 310–330 (Schmelter *et al.,* 1996). Interestingly, the homologous domain in HVP-2 vhs (HVP-2 vhs amino acids 294–314) is relatively well conserved and maintains a tryptophan residue critical for HSV vhs:VP16 interaction. These similarities, coupled with the high degree of identity between the HVP-2 VP16 and HSV VP16 proteins (J. E. Bigger and D. W. Martin, unpublished data), suggest that the HVP-2 vhs may interact with VP16 as well. In addition, recent studies by Feng and co-workers (2001) have demonstrated that HSV-1 vhs interacts with eIF4H. Presumably, the interaction of vhs with eIF4H targets vhs to regions of translation initiation. Future studies will test the hypothesis that HVP-2 vhs interacts with eIF4H and VP16.

The importance of the vhs protein for the pathogenesis of the virus has been underscored by a series of studies on the virulence of HSV deficient for vhs function (Becker *et al.,* 1993; Strelow and Leib, 1995; Strelow *et al.,* 1997; Smith *et al.,* 2000, 2002). Basically, these studies have shown that deletion of vhs function attenuates viral infection and latency in small animal models of disease presumably due to altered patterns of viral gene expression. vhs is also likely to play an important role in viral evasion of the host immune response. Evidence for this role in immune evasion has been provided by several studies. First, vhs function has been associated with a reduction in host cell MHC class I expression which would be expected to result in a lower degree of presentation of viral antigen to the immune system (Hill *et al.,* 1994). The vhs protein of bovine herpes virus 1 has also been associated with the down-regulation of MHC class I in infected cells (Koppers-Lalic *et al.,* 2001). Second, the vhs protein of HSV-2 has been shown to inhibit the activity of $CD8^+$ CTL clones against HSV-2-infected fibroblasts (Tigges *et al.,* 1996). Third, using γ-irradiated mice, Suzutani and co-workers (2000) demonstrated that vhs function could inhibit the innate immune response. Interestingly, deletion of the vhs gene improves the immunogenicity of a replication-incompetent vaccine strain (Geiss *et al.,* 2000). Presumably, increased levels of viral gene products coupled with enhanced antigen presentation provide more target antigen to the host immune system. Taken together, it is likely that an additional function of vhs is to modulate the host immune response to infection. Future studies are planned to assess the role of HVP-2 vhs in immune evasion in a primate host.

The potential role of vhs in immune evasion makes vhs-deficient viruses attractive candidates for vaccine development. Furthermore, vhs-deficient viruses demonstrate low levels of pathogenicity in mouse models and do not establish or reactivate from latency efficiently (Strelow and Leib, 1995, 1997). Interestingly, a vaccine strategy that employed a replication-defective virus with a deletion in ICP8 demonstrated greater levels of efficacy when an additional mutation was introduced to make the virus vhs-deficient (Geiss *et al.,* 2000). We have identified a ICP8 homolog in HVP-2 (J. E. Bigger and D. W. Martin, unpublished data). Deletion of this gene in the context of our vhs-deficient virus would make an attractive candidate to study the efficacy of this vaccine strategy in the

context of a primate host. The results of these studies may further demonstrate that vhs-deficient viruses represent effective vaccine strategies and lead to future human trials.

MATERIALS AND METHODS

Cells and virus

Vero and NIH3T3 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified essential medium (DMEM) (GIBCO Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and penicillin and streptomycin. Primary baboon fibroblasts were obtained by skin punch biopsy of baboons (*Papio anubis*) at Southwest Foundation for Biomedical Research under strict animal welfare standards. Briefly, skin punches $< 1\ cm^2$ were trypsinized to release fibroblasts from the tissue matrix and the cells were plated in DMEM with 10% FBS. Aliquots of low passage cells were frozen at −130°C. Cells used to detect vhs activity were used at <20 passages. HVP-2 strain 860 was obtained from R. Eberle, Oklahoma State University, Stillwater, OK. HSV-1 KOS and HVP-2 were propagated and titered on Vero cells.

Protein analysis

Cells were infected or mock-infected in duplicate at varying m.o.i. in DMEM with 10% FCS. One hour before harvesting, monolayers were washed with minimal essential medium (MEM) without methionine or cysteine (Selectamine, GIBCO). Cultures were then incubated in MEM (methionine- and cysteine-free) containing 0.1 μCi/ml EasyTag Express protein labeling mix ([^{35}S]-methionine and -cysteine, NEN Life Science Products, Boston, MA). For SDS–PAGE analysis, cells were lysed directly with Tris–glycine sample buffer (Novex) containing 2-mercaptoethanol and heated at 100°C for 5 min. Equivalent amounts of extract were run on 4–20% Tris–glycine gels (Novex). The levels of protein synthesis were visualized by autoradiography. To determine the role of the virion in vhs function, Vero cells were treated with 40 μg/ml actinomycin D (Sigma) to inhibit transcription during the course of the infection. To quantitate the amount of labeled protein, duplicate infected and mock-infected samples were scraped into phosphate-buffered saline (PBS) and subjected to three freeze-thaw samples from −70°C to 37°C. Equivalent amounts of extract were precipitated with 10% trichloroacetic acid (TCA) in the presence of 100 μg/ml bovine serum albumin (BSA). Acid precipitable counts were collected by filtration through 0.45-μm glass wool filters and quantitated by liquid scintillation counting.

Northern analysis

Vero cells were infected or mock-infected at varying m.o.i. for 4 h. RNA was isolated using the RNeasy miniprep (Qiagen, Valencia, CA) and quantitated on a DU-68 spectrophotometer (Beckman). Two to four micrograms of RNA were resolved by formaldehyde-agarose gel electrophoresis and Northern blotted using materials from the NorthenMax blotting system (Ambion, Austin, TX). Blots were hybridized with random-prime labeled mouse β-actin or GAPDH (Ambion). The level of specific message was determined by autoradiography and quantitated by phosphorImager analysis (Molecular Dynamics phosphorImager 445SI).

Sequence alignments

Sequence alignments and phylogenetic tree were conducted using the Megalign program (DNAStar, Madison, WI). A phylogenetic tree was constructed using the Clustal method of alignment with PAM250 residue weight table. Accession numbers of protein sequences from GenBank are as follows: HSV-1 KOS (AF007815), HSV-2 strain 333 (AF007816), BHV-1.1 (AJ004801), EHV-1 (M86664), PrV (S57917), GHV-2 (L40429), GHV-1 (AF168792), VZV strain Dumas (P09275), and HVP-2 strain 860 (AAG01880).

Generation of HVP-2Δvhs and HVP-2ΔvhsR

Disruption of the HVP-2 vhs gene was accomplished by marker transfer. Briefly, the HVP-2 UL41 (vhs) homolog was isolated on a 4-kb *Bam*HI clone (pBam20). The gene encoding the EGFP from the vector pEGFP-N1 (Clontech) was cloned by PCR downstream of the human cytomegalovirus (CMV) immediate-early promoter in the pCI expression vector (Promega, Madison, WI) using the restriction sites *Nhe*I and *Eco*RI to generate pCI-EGFP. The CMV-EGFP was PCR amplified using primers containing Bsu36I restriction sites at the termini so that the product could be cloned into the unique Bsu36I restriction site of pBam20 to generate pBam20-EGFP. The insertion of this fragment into the vhs gene interrupts the vhs open reading frame 60 nucleotides from the amino-terminal end of the UL41 open reading frame, thereby interrupting the vhs protein between the alanine and leucine residues at positions 20 and 21. pBam20-EGFP was transfected with wild-type HVP-2 strain 860 DNA into Vero cells. Recombinant virus plaques were selected by EGFP expression under fluorescent microscopy. The virus was plaque purified three times. Virus stocks were prepared by low multiplicity of infection passage on Vero cells. Marker transfer was used to rescue the CMV-EGFP insert from the HVP-2 vhs gene. HVP-2Δvhs DNA was cotransfected into Vero cells with pBam20. Plaques that did not exhibit EGFP expression using fluorescent microscopy were identified, picked, and subsequently plaque-purified three times. The resulting virus was designated HVP-2ΔvhsR and was grown to high titer on Vero cells. The identity of the respective viruses was confirmed relative

to wild-type virus by Southern analysis using probes specific for the vhs homolog and EGFP genes.

Characterization of HVP-2Δvhs growth *in vitro*

Plaque morphology was assessed on Vero cells with an overlay of DMEM/5% fetal calf serum/1% methyl cellulose at 2–3 days postinfection. Plaques were visualized on an Olympus CK2 microscope. Single-step growth curves were performed in duplicate on Vero cells. Briefly, 1×10^5 cells in 24-well dishes (Falcon) were inoculated with two plaque forming units per cell of wild-type or mutant virus in 200 μl of media and incubated at 37°C for 1 h. The infectious inoculum was aspirated and replaced with 1 ml of complete growth media (DMEM with 10% fetal bovine serum). At varying times postinfection samples were frozen at −70°C. Samples were thawed quickly to release infectious virus and subjected to a low-speed centrifugation to remove cellular debris. Aliquots of cleared supernatant were assayed for the presence of infectious virus by standard plaque assay on Vero cells. Determination of burst size (number of plaque forming units released per plaque) was determined on Vero cells and primary baboon fibroblasts. Briefly, cells were infected in quadruplicate on six-well dishes with increasing dilutions of virus. The virus was allowed to adhere for 1 h at 37°C. The inoculum was removed and replaced with complete growth medium (two wells) or growth medium plus methyl cellulose (two wells). After plaques were fully formed at 3–4 days postinfection, the number of plaques per well was determined from the samples containing the methyl cellulose overlay. The corresponding plates under DMEM were frozen and thawed, and the debris was pelleted by low-speed centrifugation. The number of plaque forming units in the supernatant was determined by standard plaque assay on Vero cells. The number of plaque forming units per original plaque was determined by comparing the yield with the number of plaques counted for the respective dilution relative to the number of plaques originally counted on the first series of dilutions plated under methyl cellulose.

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Note added in proof. During the publication of this manuscript Everly *et al.* (*J. Virol.* **76,** 8560–8571, 2002) have provided additional evidence that the Vhs protein is a nuclease. In addition, Keadle *et al.* (*J. Gen. Virol.* **83,** 2361–2365, 2002 and *J. Virol.* **76,** 3615–3625, 2002) have demonstrated that vhs-deficient viruses are effective therapeutic vaccines in a mouse model of ocular infection.

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